MOUSE α1-Acid Glycoprotein (AGP) ELISA KIT
96-Well Plate (EZMAGP-23K)

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Mouse α1-Acid Glycoprotein (AGP) ELISA KIT
96-Well Plate (EZMAGP-23K)

I. INTENDED USE

This Mouse AGP ELISA kit is used for the non-radioactive quantification of mouse AGP in mouse serum, plasma, tissue extract and cell culture samples. One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for research purpose only.***

II. PRINCIPLES OF PROCEDURE

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of mouse AGP molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of anti-mouse AGP polyclonal antibody, 2) wash away of unbound materials from samples, 3) binding of a biotinylated anti-mouse AGP polyclonal antibody to the captured molecules, 4) wash away of unbound materials from samples, 5) conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 6) wash away of free enzyme conjugates, and 7) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured mouse AGP in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of mouse AGP.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

A. Mouse AGP ELISA Plate

Coated with mouse AGP antibody
Quantity: 1 strip plate
Preparation: Ready to Use
Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8°C.

B. Adhesive Plate Sealer

Quantity: 2 sheets
Preparation: Ready to Use

C. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.
Quantity: 2 bottles containing 50 mL each
Preparation: Dilute 1:10 with distilled or deionized water.

III. REAGENTS SUPPLIED (continued)

D. Mouse AGP Standard

Mouse AGP, lyophilized.
Quantity: 0.5 mL/vial upon hydration
Preparation: Reconstitute with 0.5 mL distilled or deionized water. See insert for concentration.

E. Mouse AGP Quality Controls 1 and 2

One vial each, lyophilized, containing mouse AGP at two different levels..
Quantity: 0.5 mL/vial upon hydration
Preparation: Reconstitute with 0.5 mL distilled or deionized water.

F. Assay Buffer

0.05 Phosphosaline, pH 7.6 containing 0.025M EDTA, 0.08% Sodium Azide, 1.0% BSA
Quantity: 40 mL
Preparation: Ready to Use

G. Mouse AGP Detection Antibody

Pre-titered Biotinylated mouse AGP Antibody
Quantity: 12 mL
Preparation: Ready to Use

H. Enzyme Solution

Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer
Quantity: 12 mL
Preparation: Ready to Use

I. Substrate (Light sensitive, avoid unnecessary exposure to light)

3, 3’, 5, 5’-tetramethylbenzidine in buffer
Quantity: 12 mL
Preparation: Ready to Use.

J. Stop Solution (Caution: Corrosive Solution)

0.3 M HCl
Quantity: 12 mL
Preparation: Ready to Use

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8°C. For longer storage (> 2 weeks), freeze Wash Buffer, Assay Buffer, AGP Standards, Quality Controls and reconstituted Standards and Controls at ≤ –20°C. Minimize repeated freeze and thaw of the AGP Standards and Quality Controls. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

A. Sodium Azide

Sodium Azide has been added to certain reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

B. Hydrochloric Acid

Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and Pipette Tips: 10 µL - 20 µL or 20 µL - 100 µL
2. Multi-Channel Pipettes and Pipette Tips: 5 µL ~ 50 µL and 50 µL ~ 300 µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum samples, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.

   Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2°C.

   Transfer and store serum samples in separate tubes. Date and identify each sample.

   Use freshly prepared serum or aliquot and store samples at ≤ –20°C for later use. For long-term storage, keep at -70°C. Avoid freeze/thaw cycles.

2. To prepare plasma samples, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.

3. If heparin is to be used as an anticoagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

4. Avoid using samples with gross hemolysis or lipemia.

VIII. SAMPLE PREPARATION

1. A 1:3000 sample dilution is required for serum or plasma samples.
   a. 10 µL of neat sample with 990 µL of assay buffer for a 1:100 dilution
   b. 30 µL 1:100 diluted sample with 870 µL of assay buffer for a 1:3000 dilution to be used in the assay.

2. Tissue extracts or cell culture media samples may require dilution. Dilutions should be performed using the assay buffer provided.

IX. REAGENT PREPARATION

A. Mouse AGP Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Mouse AGP Standard with 0.5 mL distilled or deionized water into the glass vial to give a concentration described in the analysis sheet.. Invert and mix gently and let sit for 5 minutes then mix well.

2 Label six tubes, 1, 2, 3, 4, 5 and 6. Add 200 µL Assay Buffer to each of the six tubes. Perform 3 time serial dilutions by adding 100 µL of the reconstituted standard to Tube 1, mix well and transfer 100 µL from Tube 1 to Tube 2, mix well and transfer 100 µL from Tube 2 to Tube 3, mix well and transfer 100 µL from Tube 3 to Tube 4, mix well and transfer 100 µL from Tube 4 to Tube 5, mix well and transfer 100 µL from Tube 5 to Tube 6 and mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at ≤ -20℃. Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration pg/mL
0.5 mL	0	X (refer to analysis sheet for exact concentration)

Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add	Standard Concentration (pg/mL)
1	200 µL	100 µL of reconstituted Standard	X/3
2	200 µL	100 µL of Tube 1	X/9
3	200 µL	100 µL of Tube 2	X/27
4	200 µL	100 µL of Tube 3	X/81
5	200 µL	100 µL of Tube 4	X/243
6	200 µL	100 µL of Tube 5	X/729

B. Mouse AGP Quality Control 1 and 2 Preparation

1. Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Mouse AGP Quality Control 1 and Quality Control 2 with 0.5 mL distilled or deionized water into the glass vials. Invert and mix gently, let sit for 5 minutes then mix well.

X. ASSAY PROCEDURE

Pre-warm all reagents to room temperature prior to setting up the assay.

1. Dilute the 10X concentrated Wash Buffer 10 fold by mixing the entire contents of each bottle of wash buffer concentrate with 450 mL deionized or distilled water (dilute both buffer bottles with 900 mL deionized or distilled water).

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and wash each well 3 times with 300 µL of diluted wash buffer. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. **Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, use a gentle wash program for all washing steps described in this protocol.**

3. Add in duplicate, 80 µL Assay Buffer to the blank wells, Standard wells, QC1 and QC2 wells, and sample wells.

4. Add in duplicate, 20 µL Assay Buffer in the Blank wells, 20 µL Mouse AGP Standards in the order of ascending concentration to the appropriate wells. Add in duplicate, 20 µL QC1 and 20 µL QC2 to the appropriate wells. Add sequentially, 20 µL of the unknown samples (1:3000 dilution with Assay Buffer is required for serum or plasma samples) in duplicate to the remaining wells. **For best result all additions should be completed within 30 minutes.**

5. Cover the plate with plate sealer and incubate at room temperature for 1.5 hours on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400 to 500 rpm.

6. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.

7. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

8. Add 100 µL Detection Antibody to all wells. Cover the plate with plate sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400 to 500 rpm.

9. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.

10. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

11. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

12. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.

X. ASSAY PROCEDURE (continued)

13. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

14. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for 5 to 20 minutes (A longer development time may be needed if using a plate washer). Blue color should be formed in wells of Mouse AGP standards with intensity proportional to increasing concentrations of AGP.

Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

15. Remove sealer and add 100 µL Stop Solution **[CAUTION: CORROSIVE SOLUTION]** and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units. The absorbance of the highest AGP standard should be approximately 2.-3.2, or not to exceed the capability of the plate reader used.

Assay Procedure for Mouse AGP ELISA kit (EZMAGP-23K)

	Step 1	Step 2	Step 3	Step 4	Step 5-7	Step 8	Step 9-10	Step 11	Step 12-13	Step 14	Step 14	Step 15	Step 15
Well #	**Dilute each bottle of 10X Wash Buffer with 450 mL Deionized Water.**	Wash each well 3 times with 300 µL of diluted wash buffer **Remove residual buffer by tapping smartly on absorbent towels**	Assay Buffer	Buffer/ Standards/ Controls/ Samples	**Seal, Agitate, Incubate 1.5 hour at Room Temperature. Wash 3X with 300 µL Wash Buffer**	Detection Ab	**Seal, Agitate, Incubate 1 hour at Room Temperature. Remove residual buffer by tapping smartly on absorbent towels. Wash 3X with 300 µL Wash Buffer**	Enzyme Solution	**Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 3X with 300 µL Wash Buffer**	Substrate	**Seal, Agitate, Incubate 5-20 minutes at Room Temperature.**	Stop Solution	**Read Absorbance at 450 nm and 590 nm.**
A1, B1			80 µL	20 µL of Assay Buffer		100 µL		100 µL		100 µL		100 µL	
C1, D1			↓	20 µL of Tube 6 Std		↓		↓		↓		↓	
E1, F1				20 µL of Tube 5 Std									
G1, H1				20 µL of Tube 4 Std									
A2, B2				20 µL of Tube 3 Std									
C2, D2				20 µL of Tube 2 Std									
E2, F2				20 µL of Tube 1 Std									
G2, H2				20 µL reconstituted Standard									
A3, B3				20 µL of QC1									
C3, D3				20 µL of QC2									
E3, F3				20 µL of Sample									
G3, H3 ↓				20 µL of Sample									

XI. MICROTITER PLATE ARRANGEMENT

Mouse AGP ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 3 Std	QC 1	Sample 3								
B	Blank	Tube 3 Std	QC 1	Sample 3								
C	Tube 6 Std	Tube 2 Std	QC 2	Sample 4								
D	Tube 6 Std	Tube 2 Std	QC 2	Sample 4								
E	Tube 5 Std	Tube 1 Std	Sample 1	Sample 5								
F	Tube 5 Std	Tube 1 Std	Sample 1	Sample 5								
G	Tube 4 Std	Reconst. Standard	Sample 2	Etc								
H	Tube 4 Std	Reconst. Standard	Sample 2	Etc								

XII. CALCULATIONS

The dose-response curve of this assay fits best to a 4 or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4 or 5-parameter logistic function. The serum or plasma sample data need to be multiplied by 3000 (dilution factor) to obtain final sample concentration.

Note: When sample volumes assayed differ from 20 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 5 µL of sample is used, then calculated data must be multiplied by 4). When sample volume assayed is less than 20 µL, compensate the volume deficit with assay buffer.

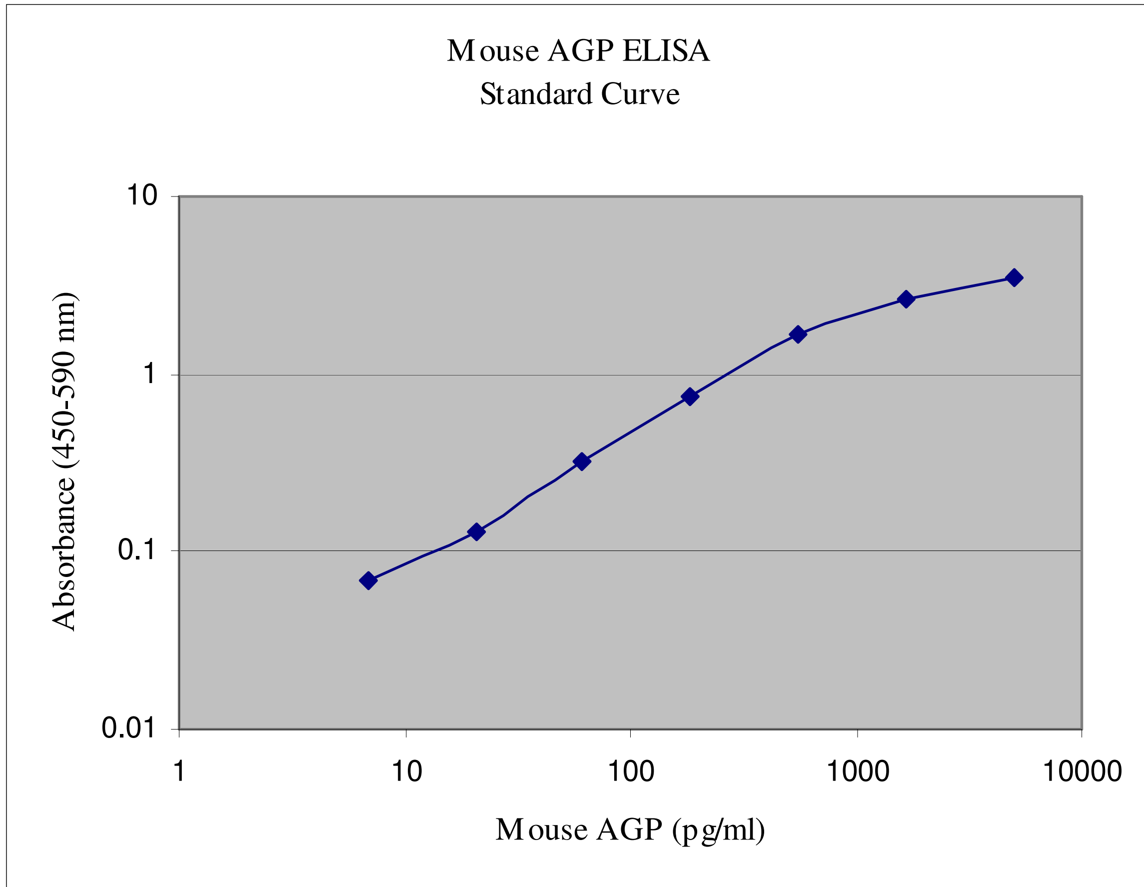
XIII. INTERPRETATION

A. Acceptance Criteria

1. The assay will be considered accepted when all Quality Control values fall within the calculated Quality Control Range. If any QC's fall outside the control range, review results with a supervisor.
2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.
3. The limit of sensitivity of this assay 6.9 pg/mL mouse AGP (20 µL sample size).
4. The appropriate range of this assay 6.9 pg/mL to 5000 pg/mL mouse AGP (20 µL sample size). Any result greater than 5000 pg/mL in a 20 µL sample should be diluted using Assay Buffer, and the assay repeated until the results fall within range. Tissue/cell extracts or cell culture media samples greater than 5000 pg/mL in a 20 µL sample should be diluted in Assay Buffer.

XIV. STANDARD CURVE

Typical Standard Curve, not to be used to calculate results.



XV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Mouse AGP that can be detected by this assay is 6.9 pg/mL when using a 20 µL sample size.

B. Specificity

The antibody pair used in this assay is specific to mouse AGP and has no significant cross-reactivity with other mouse acute phase proteins or Adipokines.

C. Precision

Intra-Assay Variation

Sample No.	Mean AGP Levels (pg/mL)	Intra-Assay %CV
1	273	7%
2	1923	8%

Inter-Assay Variation

Sample No.	Mean AGP Levels (pg/mL)	Inter-Assay %CV
1	248	4%
2	1757	10%

The assay variations of Millipore Mouse AGP ELISA kits were studied on two samples at two levels on AGP standard curve. The mean intra-assay variation was calculated from results of eight determinations of the indicated samples. The mean inter-assay variations of each sample was calculated from results of six separate assays with duplicate samples in each assay.

XV. ASSAY CHARACTERISTICS (continued)

D. Recovery

Spike & Recovery of Mouse AGP in Serum

Sample No.	AGP Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	% of Recovery
1				
	0	714	714	
	123	837	819	98%
	370	1084	862	80%
2				
	0	612	612	
	123	735	714	97%
	370	982	801	82%
3				
	0	584	584	
	123	707	696	98%
	370	954	866	91%
4				
	0	600	600	
	123	723	710	98%
	370	970	787	81%
5				
	0	447	447	
	123	570	493	86%
	370	817	666	82%

Varying amounts of mouse AGP were added to five mouse serum samples and the AGP concentration was determined. The % of recovery = observed AGP concentrations/expected AGP concentrations x 100%.

XV. ASSAY CHARACTERISTICS (continued)

E. Linearity

Effect of Serum Dilution

Sample No.	Volume Sampled (µL)	Expected (pg/mL)	Observed (pg/mL)	% Of Expected
1	20	627		
	6.67	209	192	92%
	2.22	70	66	95%
	0.74	23	26	112%
2	20	542		
	6.67	181	181	100%
	2.22	60	57	95%
	0.74	20	24	120%
3	20	704		
	6.67	235	214	91%
	2.22	78	74	95%
	0.74	26	25	107%
4	20	491		
	6.67	164	152	93%
	2.22	55	52	95%
	0.74	18	22	121%

Four mouse serum samples with the indicated sample volumes were assayed. Required amounts of assay buffer were added to compensate for lost volumes below 20 µL. The resulting dilution factors of neat, 3, 9, and 27 representing 20 µL, 6.67 µL, 2.22 µL, and 0.74 µL sample volumes assayed, respectively, were applied in the calculation of observed AGP concentrations. % expected = observed/expected x 100%.

XVI. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com.

XVII. TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. Do not let the absorbency reading of the highest standard reach 3.0 units or higher after acidification.
9. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with Wash Buffer or 3) overexposure to light after substrate has been added.

XVIII. REPLACEMENT REAGENTS

Reagents	Cat. #
Mouse AGP ELISA Plate	EP23
10X HRP Wash Buffer Concentrate	EWB-HRP
Mouse AGP Standards	E8023-K
Mouse AGP Quality Controls 1 and 2	E6023-K
Assay Buffer	AB-P
Mouse AGP Detection Antibody	E1023
Enzyme Solution	EHRP-3
Substrate	ESS-TMB3
Stop Solution	ET-TMB

XIX. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:
Toll Free US (866) 441-8400
(636) 441-8400

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers in placing an order or obtaining additional information about Millipore products, please contact your distributor or sales representative, or email our European Customer Service at customerserviceEU@Millipore.com.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to human or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.